

Review Article**Detection of Minimal Residual Disease in Acute Leukemia by Flow Cytometry****Dario Campana^{1,2*} and Elaine Coustan-Smith¹**¹Departments of Hematology-Oncology, and Pathology and Laboratory Medicine, St. Jude Children's Research Hospital, Memphis, Tennessee²Department of Pediatrics, University of Tennessee College of Medicine, Memphis, Tennessee

Patients with acute leukemia in clinical remission may still have up to 10^{10} residual malignant cells (the upper limit of detection by standard morphologic techniques). Sensitive techniques to detect minimal residual disease (MRD) may allow better estimates of the leukemia burden and help the selection of appropriate therapeutic strategies. Flow cytometry and polymerase chain reaction have emerged as the most promising methods for detecting submicroscopic levels of leukemia. Flowcytometric detection of MRD is based on the identification of immunophenotypic combinations expressed on leukemic cells but not on normal hematopoietic cells. It affords the detection of one leukemic cell among 10,000 normal bone marrow cells, and can be currently applied to at least two thirds of all patients with acute leukemia. Prospective studies in large series of patients have demonstrated a strong correlation between MRD levels during clinical remission and treatment outcome. Therefore, MRD assays can be reliably used to assess early response to treatment and predict relapse. In this review, we discuss methodologic aspects and clinical results of flowcytometric detection of MRD in patients with acute leukemia. *Cytometry (Comm. Clin. Cytometry)* 38: 139–152, 1999. © 1999 Wiley-Liss, Inc.

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BIOLOGY AND TREATMENT OF ACUTE LEUKEMIA

In the modern treatment of acute leukemia, advances in supportive care have allowed the intensification of therapy, and survival rates have risen accordingly. Cure rates in childhood acute lymphoblastic leukemia (ALL) have increased from 10% to 80% over the last four decades (1). There has also been progress, although less dramatic, in curing acute myeloid leukemia (AML) in children and ALL and AML in adults (2–4). However, as therapy is intensified, the risk of serious sequelae such as secondary leukemias, brain tumors, cardiomyopathy, and neuropsychological problems increases (1, 5–9). Clearly, further intensification of treatment for all patients is not an appropriate strategy.

It has become evident that each subgroup of leukemias has heterogeneous biologic features, many of which are associated with responses to therapy (10). For example, children with ALL who have genetic features such as the chromosomal translocation t(9;22) or rearrangements of the *MLL* gene respond poorly to chemotherapy (2); often, bone marrow transplantation is the only curative option for these patients. Conversely, children whose leukemic blast cells contain 51 to 65 chromosomes or *TEL* rearrangements respond consistently well to chemotherapy (11, 12). Similar distinctions have been delineated in AML:

cases with 16q22 abnormalities, t(15;17), or t(8;21) have a more favorable prognosis than those with 11q, 5q or 7q abnormalities or hypodiploidy (13). Because of their correlation with outcome, these features are currently used, together with clinical parameters such as white blood cell counts and age, for treatment stratification. That is, in efforts to maximize the efficacy and minimize the toxicity of treatment for leukemia, the intensity of therapy is proportional to the likelihood of relapse. Unfortunately, none of the clinical and biologic parameters currently used for risk classification is ideal. A proportion of patients with “good risk” features relapse, while others may receive more intensive treatment than is necessary. Studies of minimal residual disease (MRD) aim at improving estimates of the total burden of leukemic cells during clinical remission. This information provides an indicator of the aggressiveness and drug sensitivity of the disease,

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Table 1
Potential Applications of MRD Studies in the Clinical
Management of Acute Leukemia

Time of study	Objective
During remission induction	Measurement of early response to treatment
End of remission induction, early continuation	Identification of patients at a higher risk of relapse
Throughout treatment	Detection of impending relapse
Throughout treatment	Identification of leukemic cells in extramedullary sites (e.g., central nervous system)
Before autologous stem cell graft	Detection of contaminating leukemic cells
Before autologous stem cell graft	Evaluation of the efficacy of "purging" techniques

and helps in the selection of appropriate therapeutic strategies.

MINIMAL RESIDUAL DISEASE

Acute leukemia is generally considered to be in remission when neoplastic cells compose less than 5% of the bone marrow cell population (the lower limit of morphologic detection). However, patients with acute leukemia may have approximately 10^{12} malignant cells at diagnosis; those in remission by this criterion may have as many as 10^{10} undetectable neoplastic cells (14).

The potential applications of MRD studies in the clinical management of acute leukemia include early identification of patients at a higher risk of relapse and detection of impending clinical relapse (Table 1). In addition, MRD studies provide a powerful tool for assessing bone marrow or peripheral blood that has been harvested for autologous hematopoietic stem cell transplantation, and for determining the efficacy of "purging" procedures (15). Finally, they may serve to unequivocally demonstrate leukemic involvement of the central nervous system (16, 17).

Monitoring of MRD in patients could provide useful information on the biology of acute leukemia and its responsiveness to treatment. For example, MRD assays may be more accurate than conventional methods for measuring the rate at which cytotoxic regimens eliminate leukemic cells. Thus, MRD measurements could be used as endpoints to rapidly compare the effectiveness of different chemotherapeutic regimens.

Numerous methods of monitoring MRD in acute leukemia have been developed (14). Leukemic cells can be distinguished from normal hematopoietic cells on the basis of chromosomal or molecular abnormalities, antigen-receptor gene rearrangements, and immunophenotype. The following sections review the methodologic and clinical advances in the immunologic detection and measurement of MRD in acute leukemia.

METHODOLOGIC ISSUES IN IMMUNOLOGIC DETECTION OF MINIMAL RESIDUAL DISEASE Identification of Leukemic Cells

Early attempts of studying MRD immunologically were hampered by the limited availability of antibodies to

leukemic cell markers. It quickly became apparent that the known markers were also expressed on normal lymphohematopoietic cells. For example, terminal deoxynucleotidyl transferase (TdT) and CD10 are expressed on normal B cell precursors and on most leukemic lymphoblasts (18–22). TdT⁺, CD10⁺ cells are particularly abundant in the bone marrow of young children and in bone marrow regenerating after chemotherapy or transplantation, where they may represent 10% to 20% of the mononucleated cells (22, 23). Although the levels of TdT and CD10 expression may be abnormal in some leukemic cases (see below), these markers cannot be used alone to identify leukemic cells in the bone marrow. TdT⁺, CD10⁺ cells are extremely rare in peripheral blood (24), and early studies suggested that an increase above 0.1% in the proportion of peripheral blood TdT⁺ cells could be taken as an early sign of relapse (25). However, subsequent studies showed that the proportion of TdT⁺ cells in peripheral blood often rises in patients who remain in remission (26). CD10 has also proved to be unsuitable as a single marker for reliable MRD surveillance in peripheral blood (27).

Although the immunophenotypes of most leukemic cells are identical to those of their normal counterparts, there are notable exceptions. For example, the gene fusions which accompany chromosomal translocations such as *BCR-ABL*, *E2A-PBX1*, *MLL-AF4* and *TEL-AML1* in ALL, and *AML1-ETO* and *PML-RARA* in AML, encode chimeric proteins that are genuine tumor-associated markers. Antibodies specific for these tumor markers should be useful for studies of MRD. One such reagent, which reacts with the *E2A-PBX1* chimeric protein, has recently been described (28).

It is also possible that the leukemic process dysregulates gene expression in such a way that cellular molecules are over- or under-expressed. Two potentially useful monoclonal antibodies that react with molecules expressed by leukemic lymphoblasts but not with normal lymphoid progenitors were recently developed. One, "7.1", reacts with the human homolog of the rat chondroitin sulfate proteoglycan (NG2). It appears to specifically recognize leukemic cells defined by 11q23 abnormalities and to be absent from normal hematopoietic cells (29, 30). In our preliminary studies, this antibody was sufficiently sensitive to detect one leukemic cell among 10,000 normal mononucleated hematopoietic cells by flow cytometry (E. Coustan-Smith, D. Campana, F.G. Behm, unpublished data). Another reagent, "KOR-SA3544" has been reported to recognize leukemic lymphoblasts with *BCR-ABL* rearrangements (31). In our experience, approximately one-third of ALL cases react strongly with KOR-SA3544, including those that do not have *BCR-ABL* rearrangements (E. Coustan-Smith, D. Campana, unpublished data). Because this antibody also reacts with myeloid cells, it must be used in combination with reagents that identify lymphoid progenitors.

Current immunologic strategies for detecting residual disease rely on combinations of leukocyte markers normally not seen in peripheral blood and bone marrow (14). Such leukemia-associated phenotypes can be identified by double-, triple-, or quadruple-color staining techniques

Table 2
Immunophenotypic Combinations Used to Study MRD in ALL

ALL lineage	Leukemia-associated differences ^a	Phenotype	Frequency (%) ^b
B-lineage	Mostly quantitative	CD19/CD34/CD10/TdT	30–50
		CD19/CD34/CD10/CD22	20–30
		CD19/CD34/CD10/CD38	30–50
		CD19/CD34/CD10/CD45	30–50
	Mostly qualitative	CD19/CD34/CD10/CD13	10–20
		CD19/CD34/CD10/CD15	5–10
		CD19/CD34/CD10/CD33	5–10
		CD19/CD34/CD10/CD65	5–10
		CD19/CD34/CD10/CD21	5–10
		CD19/CD34/CD10/CD56	5–10
		CD19/CD34/CD10/KORSA3544	10–20
		CD19/CD34/TdT/cytoplasmic μ	10–20
		CD19/7.1	3–5
		CD19/p53	3–5
T-lineage	Qualitative	TdT/CD3	90–95
		CD34/CD3	30–50

^aDifferences between leukemic and normal cells in immunophenotypic expression may reflect differences in intensity of expression of one or more markers ("quantitative") or the expression of markers not found on normal lymphoid progenitors ("qualitative").

^bProportion of childhood ALL cases in which one leukemic cell in 10^4 normal bone marrow cells can be detected by using the listed immunophenotypic combination. Most cases express more than one suitable combination.

performed with antibodies conjugated to different fluorochromes. Additional capabilities of flow cytometry, such as cell sorting followed by fluorescence in-situ hybridization (FISH) (32), or simultaneous analysis of phenotype and DNA content (33), may also contribute to MRD studies.

Differences in the antigenic expression of leukemic cells and normal progenitor cells may be qualitative, quantitative, or both (Tables 2 and 3). Qualitative differences are seen in immunophenotypic combinations expressed by leukemic cells but extremely rarely by normal bone marrow cells. Examples are CD34/CD19/CD21, which is expressed in a proportion of B-lineage ALL cases, and CD34/CD56, which is expressed in some cases of AML. Normal cells with these phenotypes are encountered on rare occasions, but antigen expression is usually weak, and is well below the levels seen in leukemic blasts cells (14). The combination CD3/TdT, which is expressed by most T-ALL cells, is never observed among normal bone marrow cells (Fig. 1), although it is expressed by developing T cells in the thymus (34). Quantitative differences in antigen expression can also be used to distinguish leukemic blast cells from subsets of normal cells with similar phenotypes. For example, the expression of CD19, CD10 and CD34 in some cases of B-lineage ALL can be more than 10-fold greater than that of normal B cell progenitors (35) (E. Coustan-Smith and D. Campana, unpublished data). Underexpression of CD45 and CD38 is also an abnormal feature in some B-lineage ALL cases (36) (E. Coustan-Smith and D.

Table 3
Immunophenotypic Combinations Used to Study MRD in AML

Phenotype ^a	Frequency (%) ^b
CD13/CD33/CD34/CD11b	10–15
CD13/CD33/CD34/CD65	10–20
CD13/CD33/CD34/CD15	10–20
CD13/CD33/CD34/CD56	10–15
CD13/CD33/CD34/CD38	10–15
CD33/CD34/CD117/CD13	20–40
CD33/CD34/CD117/CD15	20–40
CD33/CD34/CD117/HLA-DR	10–15
CD33/CD34/CD117/CD11b	10–20
CD13/CD33/CD34/CD19	5–10
CD13/CD33/CD34/CD2	5–10
CD13/CD33/CD34/CD7	10–20

^aThese phenotypic combinations reflect mostly quantitative differences between leukemic myeloblasts and normal bone marrow cells in antigen expression.

^bProportion of childhood AML cases in which one leukemic cell in 10^{-3} – 10^4 normal bone marrow cells can be detected using the listed immunophenotypic combination. Most cases express more than one suitable combination.

Campana, unpublished data). Other markers, such as CD45RA, CD11a, and CD44 may also be overexpressed or underexpressed (37). Thus, dot plots of selected combinations of markers will contain areas that are consistently blank when normal bone marrow cells are analyzed (Fig.1). Positive readings in these areas will suggest residual leukemia.

To monitor residual disease, it is essential to have detailed information about the immunophenotypic features of the patient's leukemic cells at the time of diagnosis. These dictate the selection of the appropriate markers. If the immunophenotype were not known, one would have to apply the full range of potentially useful markers— an expensive and time-consuming option that might still fail to identify residual disease.

The proportion of cases that can currently be monitored for MRD by flow cytometry varies from laboratory to laboratory. Factors that influence this variability include the number of markers tested, the inclusion of bone marrow samples regenerating after chemotherapy to define the normal range, and the stringency with which the laboratory defines leukemia-associated immunophenotypes. We use only immunophenotypes that allow us to detect one leukemic cell in 10^4 or more normal cells. Obviously, if a lesser sensitivity was considered acceptable, a greater number of marker combinations could be used for MRD detection.

Tables 2 and 3 summarize the phenotypic combinations currently used in our laboratory. In virtually all cases of T-ALL, the combination of nuclear TdT with T-cell markers, such as cytoplasmic or surface CD3 or CD5, is highly informative. In cases in which TdT expression is weak or negative, CD34 can be used instead if found to be expressed at diagnosis. CD19 and MHC-Class II, which are not usually expressed on T-ALL blasts but are strongly positive on most normal bone marrow TdT⁺ cells, can be

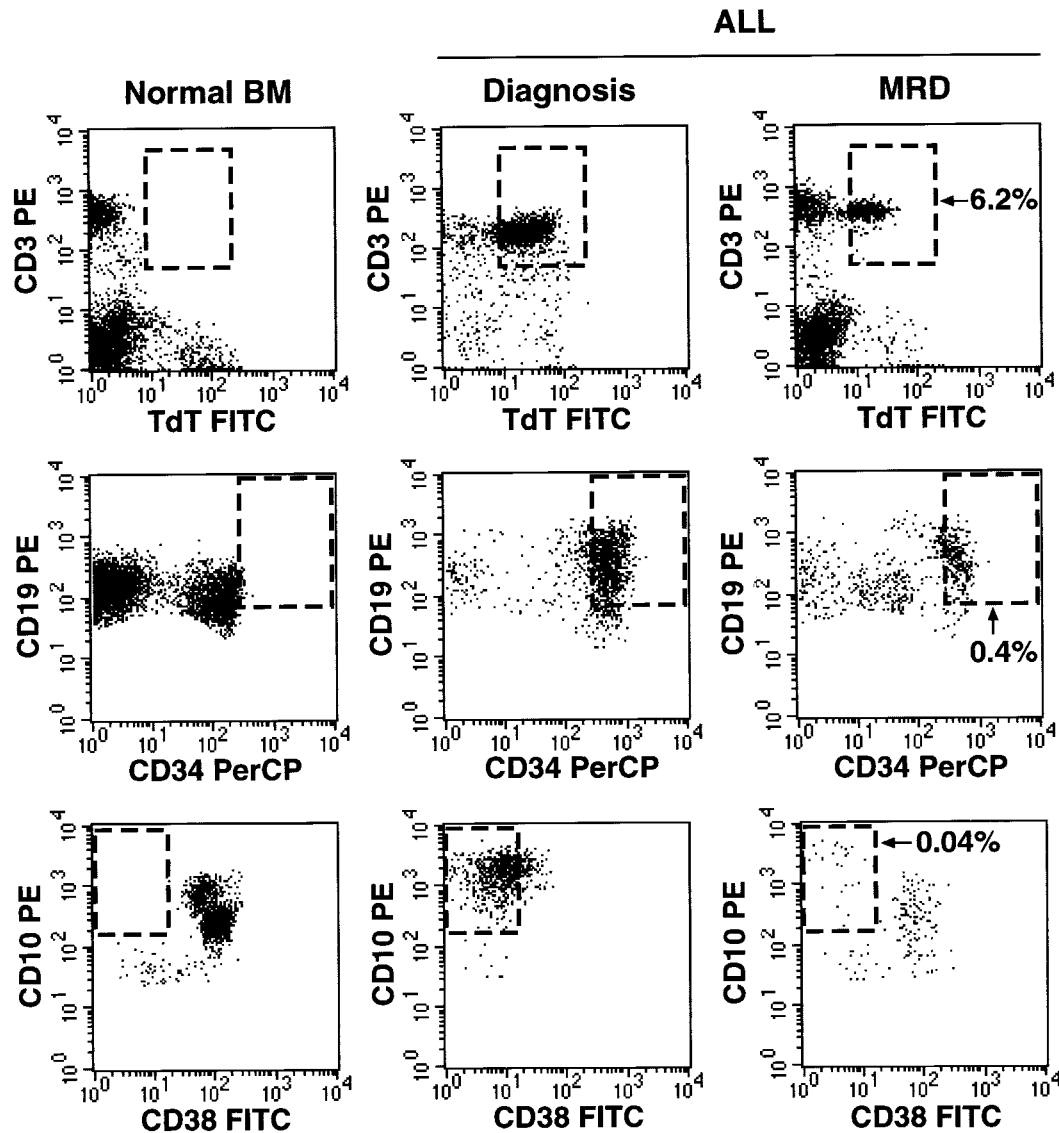


Fig. 1. Differences in antigen expression between normal and leukemic cells. Dot plots illustrate staining of normal bone marrow (BM) mononucleated cells and cells from patients with T-lineage ALL (upper panels) and B-lineage ALL (middle and lower panels) with CD3 PE and anti-TdT FITC, CD19 PE and CD34 PerCP, and CD10 PE and CD38 FITC, respectively. In the B-lineage ALL cases only CD19⁺ cells are shown. Dashed rectangles enclose areas which are consistently blank in normal BM samples. In the three ALL cases at diagnosis, most blast cells fell into these areas. Residual leukemic cells were still detectable in all three patients during clinical remission, 6 weeks after diagnosis (right panels). Levels of MRD in the B-lineage ALL cases were confirmed by PCR analysis of Ig genes. The number of events acquired in the study of normal BM samples was 10,000, 156,000 and 143,000 for the CD3/TdT, CD19/CD34 and CD10/CD38 combinations, respectively. In the study of the MRD samples it was 10,000, 112,000 and 129,000, respectively.

used to complement these markers. Thus, they can further help to distinguish normal from leukemic cells.

Detection of MRD in B-lineage ALL requires a larger panel of antibodies. We usually identify immature B cells by the simultaneous expression of CD19, CD10, and CD34 or TdT. Quantitative differences in antigenic expression between leukemic and normal cells in the expression of these markers can be used in approximately 30–50% of cases. Other useful markers whose expression may differ quantitatively in leukemic and normal immature B cells are CD38, CD45, and CD22 (E. Coustan-Smith and D. Campana, unpublished data). Qualitative differences between

normal and leukemic cells can be detected by using antibodies to myeloid- and NK-associated molecules or to molecules expressed by mature but not immature normal B cells. KOR-SA3544 and 7.1, mentioned above, are two additional informative markers. With all these marker combinations, approximately 85% of B-lineage ALL cases can be studied at the 1 in 10⁴ level of sensitivity.

Abnormal expression of cell markers reportedly occurs in more than two thirds of AML cases (38–41). It is not clear whether all of these abnormal immunophenotypes can be used for sensitive detection of MRD. The CD34/CD56 combination appears to be one of the most useful

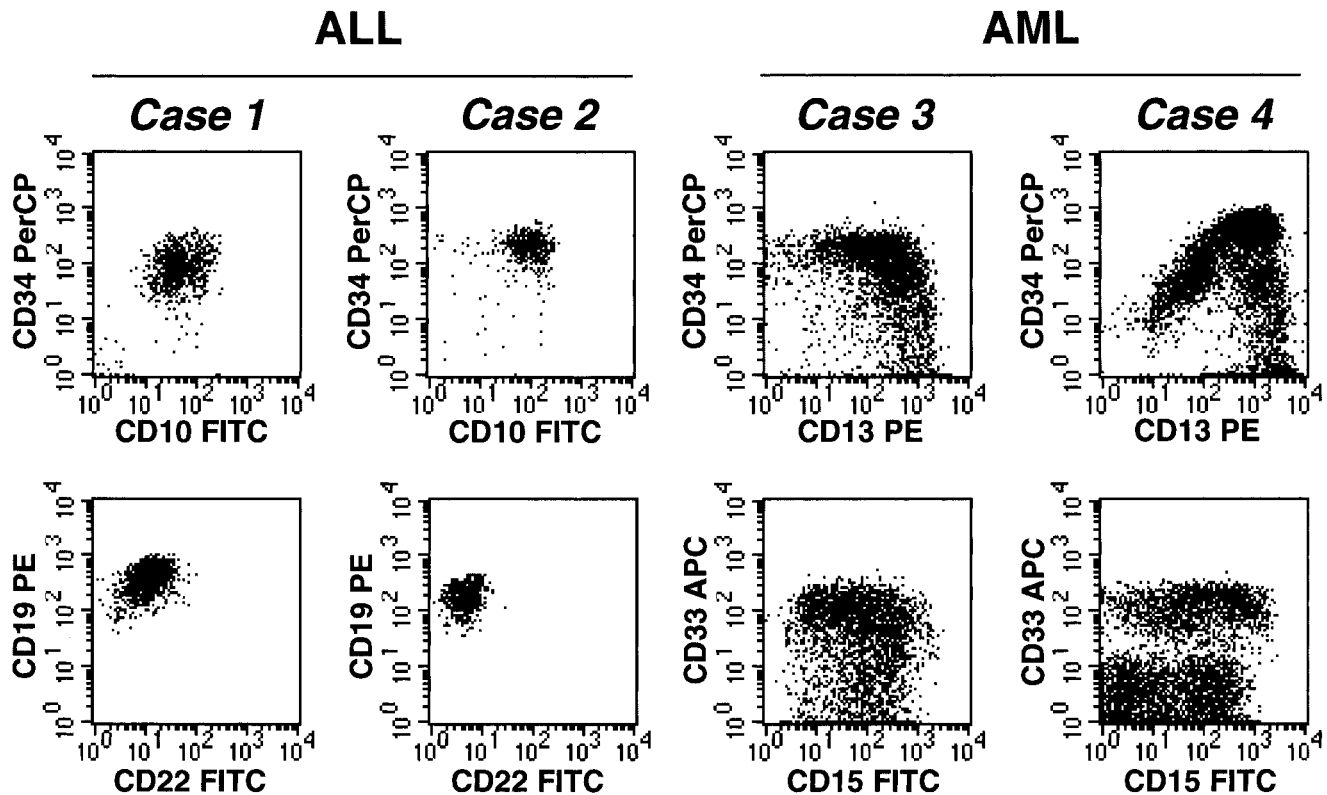


Fig. 2. Differences in phenotypic expression between ALL and AML. Dot plots illustrate diagnostic samples from two patients with ALL and two patients with AML. In general, leukemic lymphoblasts are immunophenotypically more homogeneous than leukemic myeloblasts.

for this purpose (42). CD34 and CD56 are simultaneously expressed in approximately 20% of childhood AML cases, and this phenotype is associated with the t(8;21)(q22;q22) (43). Although AML patients with this translocation generally respond better to chemotherapy, those whose blast cells express CD56 have a distinctively poorer outcome (44). Monitoring MRD at 10^{-4} sensitivity level with this combination is possible (42). Another recently published marker combination that may be useful for MRD detection is CD34 and CD87; 70% to 80% of AML cases are reportedly CD87⁺, but the marker is found in only <0.2% of normal CD34⁺ cells (45). In our experience, approximately 60% of cases of childhood AML can currently be monitored for MRD with a sensitivity of 10^{-3} to 10^{-4} (Table 3). However, the immunophenotype of AML is generally more heterogeneous than that of ALL, and it is common to observe several immunophenotypic subsets at the time of diagnosis (Fig. 2). This heterogeneity may complicate the selection of the most appropriate markers and gates to use for MRD studies.

Methods of Detecting Antibody-Bound Cells

Antibody binding to cells can be detected by a variety of methods, including immunoperoxidase, immunoalkaline phosphatase, immunogold, and immunofluorescence techniques. However, the first three methods are not highly suitable for multiple marker analysis, which is essential for MRD studies. Thus, immunofluorescence is clearly the

method of choice. Pioneering MRD studies were performed by fluorescence microscopy (46, 47). This approach is still valid, especially when high-quality fluorescence microscopes with selective blocking filters for different fluorochromes and with phase-contrast attachments are used. However, microscopic screening of a large number of cells is tedious and time consuming. The automation of this process by computerized image analysis is desirable but we have not yet encountered a sufficiently sensitive and accurate instrument that we could recommend for reliable MRD detection. Virtually all laboratories currently prefer flow cytometry, which allows multiparameter analysis, antigen quantitation, and rapid screening of large numbers of cells.

In the past, intracellular antigen expression (e.g., nuclear TdT, cytoplasmic CD3, myeloperoxidase) was optimally investigated by fluorescence microscopy of cytocentrifuge preparations (48). This method was used because membrane permeabilization methods for cells in suspension caused cell loss and greatly damaged cell structure, severely altering the cells' light-scattering patterns and precluding the distinction of morphologic subsets. With the availability of techniques and reagents for cell fixation and permeabilization that preserve cell morphology, it has become possible to detect intracellular antigens in cell suspensions by flow cytometry (49–55). In earlier studies, we used a method in which cells are fixed with 0.25% paraformaldehyde and permeabilized with 0.2% Tween 20

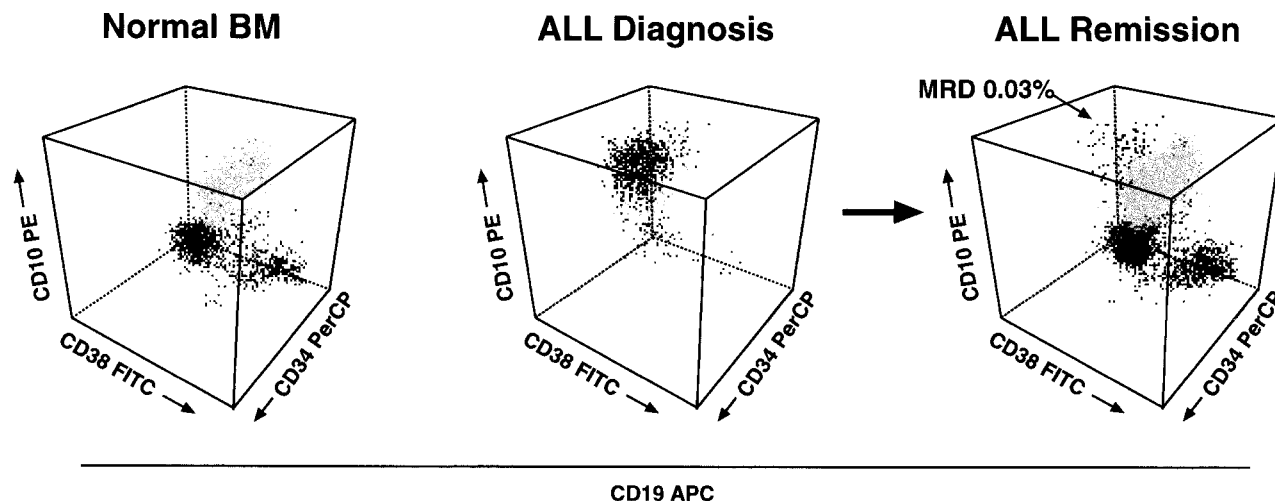


FIG. 3. Detection of MRD with 4-color flow cytometry. Normal bone marrow (BM) mononucleated cells, and BM mononucleated cells obtained from a patient with B-lineage ALL at diagnosis and at the end of remission induction (6 weeks after diagnosis) were stained with CD19 APC, CD10 PE, CD34 PerCP and CD38 FITC. The tridimensional dot plots show the immunophenotype of CD19⁺ lymphoid cells in the three samples. In the patient with ALL, MRD studies detected 0.03% of cells with the leukemia-associated phenotype, despite morphologic remission. Levels of MRD were confirmed by PCR analysis of Ig genes.

(51). Although it provided good labeling of intracellular molecules, this method altered the light-scattering properties of the cells to a certain extent. More recently, we have used a commercially available reagent (ORTHO Permea-Fix; Ortho Diagnostic Systems, Raritan, NJ) (52) with satisfactory results. Cell morphology is virtually indistinguishable from that of unpermeabilized cells, and the labeling of intracellular antigens, such as cytoplasmic CD3, cytoplasmic μ , and nuclear TdT, is also optimal. Another reagent (Fix & Perm; An der Grub, Vienna, Austria, and Caltag, South San Francisco, CA) also yields excellent labeling of intracellular antigens with minimal damage to cell morphology and allows superior staining of myeloperoxidase (53, 55, 56).

Discrimination of leukemic cells from normal hematopoietic cells by flow cytometry improves if several cellular parameters are examined. Currently, most laboratories use 3-color analysis for MRD studies, including antibodies conjugated to FITC, PE, and PerCP. Dual-laser flow cytometers that allow the detection of other fluorochromes such as APC and permit 4-color analysis appear to be ideally suited for these studies (Fig. 3). In addition, this approach potentially allows a reduction in the number of individual test tubes per sample, thus employing reagents and cells more efficiently. However, sensitivity, precision and accuracy of 4-color and 3-color analysis have not yet been formally compared.

Sensitivity and Reliability of Immunophenotypic Analysis

The sensitivity of all MRD detection methods depends on the number of cells that can be analyzed. A marrow sample taken from a typical patient with acute leukemia in "remission" yields 5×10^7 or fewer mononuclear cells, and technical constraints may limit the number of cells available for study to less than 1×10^6 . Flow cytometry allows the detection of 1 target cell in 10^6 or more cells,

providing that a large number of cells is studied (e.g., 10^7 or more) and the fluidics system is exhaustively cleansed (57–60). Because such large samples are rarely available during routine MRD studies in patients with leukemia, a more realistic sensitivity for practical applications is approximately 1 target cell in 10^4 to 10^5 cells. However, this high level of sensitivity can be achieved only by using markers that are strongly associated with leukemic cells. By relying on the cells' light-scattering as well as on their immunophenotype, one can identify morphologic subsets of hematopoietic cells with a precision equivalent to that of an expert hematopathologist interpreting a Romanovsky-stained smear (61). It is advisable to test each promising marker combination by using leukemic cells serially diluted with normal cells, to ensure that the necessary level of sensitivity (e.g., 1 in 10^4) can be achieved (Fig. 4)(42). Markers that afford lower levels of sensitivity (e.g., 1 in 10^3) can occasionally be used to clarify the nature of morphologically recognizable lymphoblasts. However, the use in clinical studies of marker combinations with different levels of sensitivity renders correlative studies of MRD results, clinical features, and treatment outcome difficult, if not impossible, to interpret accurately.

Figure 5 summarizes the flow cytometry protocol for MRD detection used in our laboratory. We first record the light-scattering and immunophenotypic features of 2000 to 10,000 cells. These data are then used to draw two gates: one surrounds dots reflecting light-scattering typical of leukemic blasts (gate 1), whereas the other encloses dots reflecting expression of particular cell markers (gate 2). By using Boolean instructions, we then identify cells that fulfill the criteria delineated by both gates. This method allows the screening of 1×10^6 or more bone marrow cells and the retention of data from only the relevant cells. To further examine the captured data, we assess morphologic characteristics of the cells expressing

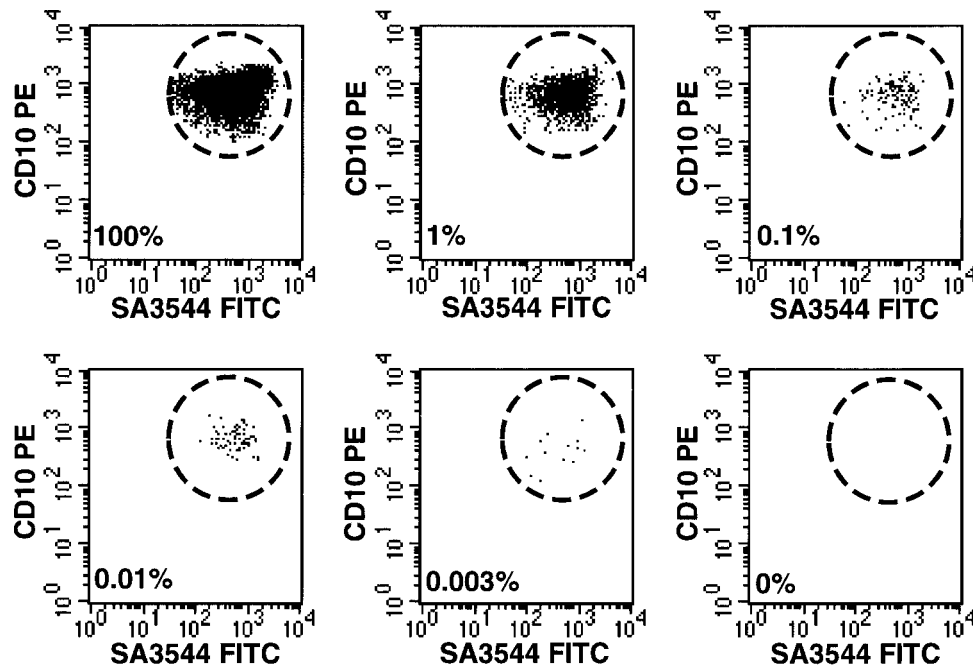


FIG. 4. Sensitivity of MRD detection by flow cytometry. ALL cells were diluted with normal peripheral blood cells at the percentages indicated. Cells were labeled with CD10 PE and KOR-SA3544 FITC. Up to 600,000 events were acquired from each cell mixture. Dashed circles enclose an area of the dot plot which is blank in normal samples but includes most leukemic blast cells in this case.

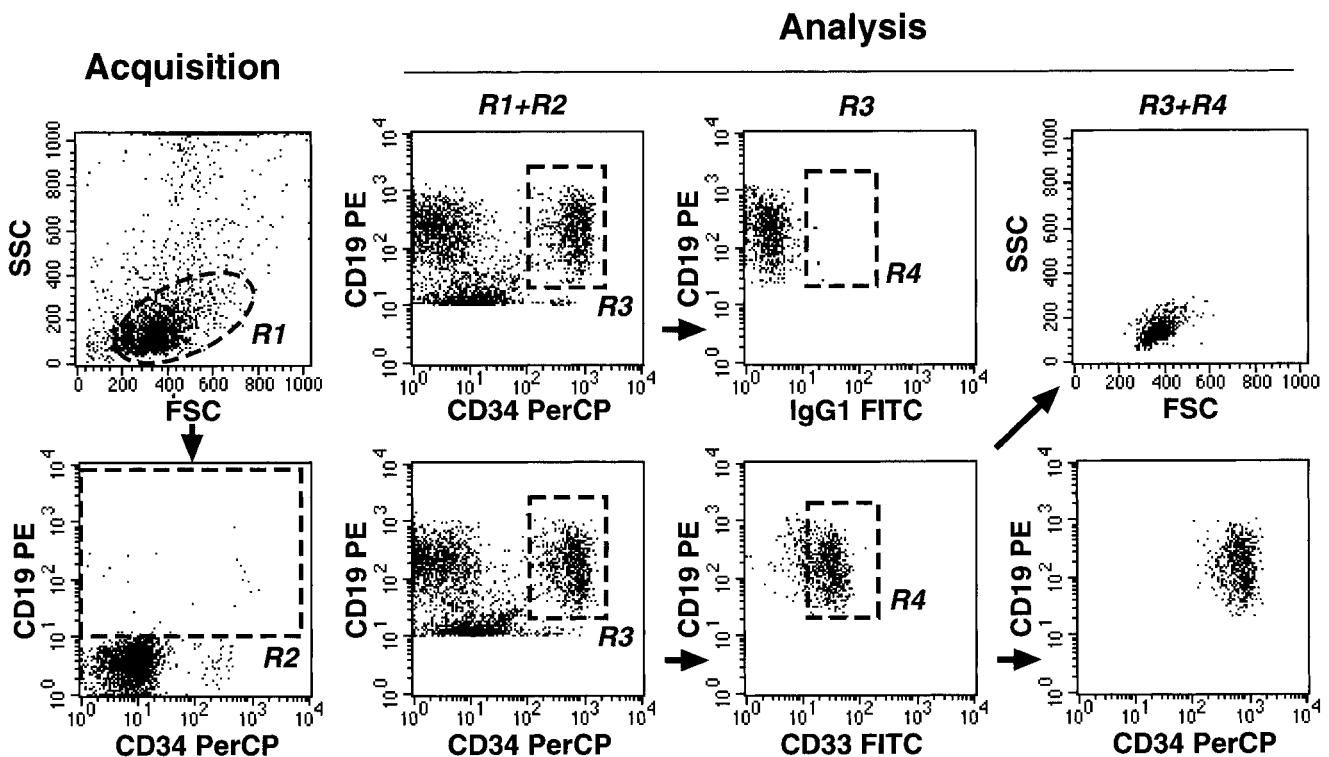


FIG. 5. Protocol for flow cytometric detection of MRD. Dot plots illustrate the study of a bone marrow sample taken from a patient with ALL during clinical remission. Leukemic cells at diagnosis expressed CD19, CD34 and CD33. Mononucleated cells from the remission bone marrow sample were labeled with CD19 PE, CD34 PerCP and CD33 FITC. In a parallel tube, CD33 FITC was replaced by nonreactive control IgG1 FITC. After analyzing 10,000 mononucleated cells, we drew two gates (far-left panels): one included cells with lymphoid morphology (R1) and the other included cells expressing CD19 (R2). We then screened 450,000 cells, retaining only the electronic events that fulfilled the criteria defined by R1 and R2 (center-left panels). The immunophenotype of CD19⁺CD34⁺ cells (R3) was analyzed for expression of CD33 (center-right panels): most cells were CD33⁺ and negative with the isotype-matched control antibody. The light-scatter and immunophenotypic features of CD19⁺CD34⁺CD33⁺ cells (R3 + R4) corresponded to those determined at diagnosis (far-right panels). Residual leukemic cells composed 0.14% of the total bone marrow mononucleated cells.

“leukemia-associated” phenotypes in the light-scattering dot plot (“back gating”), to ensure that they form a tight cluster, indicative of a homogeneous cell population. We feel that at least 10 to 20 dots must be captured in order to interpret a cluster of aberrant readings. In addition, the light-scatter profile and intensity of antigen expression should reproduce those of malignant cells characterized at the time of diagnosis. An automated analysis software such as PAINT-A-GATE[®] can also be used for this purpose. During the selection of markers suitable for MRD detection, the same procedure is applied to the analysis of normal bone marrow samples to test the specificity of the selected marker combinations.

To determine the precision of flow cytometric detection of rare cells, we prepared mixtures of leukemic and normal cells and compared the results of multiple measurements of residual leukemia in identical cell preparations. The results demonstrated that this assay is highly precise: in 23 tests of mixtures containing 1 leukemic cell in 10⁴ normal cells, results were remarkably similar (coefficient of variation = 15%); in 22 tests of mixtures containing 1 leukemic cell in 10³ cells, the coefficient of variation was 10% (62).

Technical Artifacts

Table 4 summarizes the most frequent sources of error in immunologic MRD studies. False-positive results can be caused by sample contamination in any sensitive method for MRD detection. In flow cytometry, dirty reagents or imperfect cleansing of the fluidics system can result in false-positive readings caused by residual cells or debris (57). In our laboratory, some reagents, such as permeabilization, washing and fixation solutions, are passed through a 0.22- μ m-pore filter before use. We also periodically run all fluids used in MRD detection through the flow cytometer for periods of at least 5 minutes, to detect impurities that mimic the light-scattering properties of cells. If these impurities are detected, the reagents are filtered, reanalyzed, and discarded if impurities persist. The sample capillary tube is carefully wiped after each acquisition to prevent cell carryover from one tube to the next; PBS can be used between each specimen to exclude cross-contamination. The sample capillary tube is purged daily with 1% sodium hypochlorite and the entire fluidics system is cleansed at least once a month. Finally, all reagents used for monitoring MRD are dedicated to that use and are stored separately.

False-positive results can also arise when small subsets of normal cells express immunophenotypes resembling those of ALL cells (14). Investigators interested in MRD monitoring should examine a large number of samples from normal donors in order to establish the baseline percentages and fluorescence intensities that may be expected with any given combination of antibodies. Samples from patients undergoing chemotherapy for malignancies that did not express the immunophenotypes under study at diagnosis should also be examined, including regenerating bone marrow samples with hyperactive lympho-hematopoiesis. These studies should be per-

Table 4
Methods of Avoiding Error in Immunologic MRD Studies

Problem	Result	Prevention/Solution
Sample contamination	False positive	Careful handling and labeling of samples
Impurities in washing buffers and fluidics system	False positive	Routine filtering of buffers and monitoring of their purity Cleansing of flow cytometer fluidics system
Non-specific antibody binding	False positive	Saturation of Fc receptors Careful titration of all antibodies Use of isotype-matched control antibodies Use of antibodies in F(ab') ₂ fragment form (not essential)
Antibody misplacement or contamination	False positive or negative	Orderly and logical storage of antibodies Clear labeling of test tubes Use of premixed antibody cocktails Non distracting work environment
Changes in antibody batch and/or instrument instability	False positive or negative	Frequent analysis of normal samples Periodic testing of instrument settings
Sampling from a negative site (heterogeneous distributions of leukemia)	False negative	Bone marrow sampling from several sites ^a Studies of peripheral blood ^a
Immunophenotypic shifts	False negative	Use of multiple antibody combinations

^aThe usefulness of these measures has not been formally assessed.

formed with reagents identical to the ones used for MRD monitoring (i.e., the same antibody clone conjugated to the same fluorochrome).

False positive results can be caused by using antibodies that react nonspecifically, such as those that bind cells through their Fc portion – a common feature of murine antibodies of the IgG2a isotype (63). This problem can be prevented by careful selection of optimal reagents, by saturation of Fc receptors with human or rabbit Ig, and by use of F(ab')₂ fragments rather than whole Ig molecules. In addition, isotype-matched nonreactive antibodies should be used as controls, and all antibodies should be carefully titrated.

To ensure the reliability of MRD testing, the procedures for cell collection, separation, staining and analysis must be followed meticulously. Most importantly, the sequence

in which antibodies are added to cells and the times of incubation must be rigorously standardized, because variations in these procedures can alter the intensity of cell labeling. Moreover, the reproducibility of MRD analysis can be affected by changes between different batches of antibodies and by instrument instability. These variations must be monitored by frequent staining of normal samples and by regular testing of the instrument settings.

In addition to the above potential limitations specific to flowcytometric detection of MRD, other factors may affect any method of MRD detection. For example, the anatomic distribution of leukemic cells during clinical remission may be uneven (64–66), leading to sampling variability and false-negative results.

LINEAGE SWITCHES AND IMMUNOPHENOTYPIC SHIFTS

A small fraction of patients have a recurrence of acute leukemia whose cellular features are unlike those determined at diagnosis. In the majority of cases, these leukemias are unrelated to the original leukemic clone and represent secondary malignancies, which are often caused by the mutagenic effects of leukemia treatment (6, 67). Clearly, secondary leukemias cannot be anticipated by currently available methods for monitoring MRD.

In a proportion of cases, recurrent leukemia has genetic features that confirm its relationship to the original leukemic clone, but has the phenotype of a different lineage (lineage switch). There have been reports of leukemias morphologically and immunophenotypically characterized as ALL that relapse as AML (or vice versa) while retaining the karyotypic and molecular features of the original clone (68–70). Such “lineage switch” relapses may be detected early by molecular methods such as polymerase chain reaction (PCR) amplification of chromosomal breakpoints or antigen-receptor gene rearrangements, but not by immunologic methods.

Another cause of false-negative results during monitoring for residual leukemia is clonal evolution during and after treatment, which may cause the disappearance of one or more of the markers detected at diagnosis – a phenomenon already noticed in early studies (71–73). Pui et al. (74) described loss of CD10 in 8 of the 51 “common” ALL cases studied in their series, although three of these cases had relapsed as AML. TdT was lost in 4 of 25 cases, 2 of which had relapsed as AML. Raghavachar et al. (75) studied 11 cases of ALL at diagnosis and relapse, reporting loss of CD1 and CD2 expression in one case, loss of TdT in another, and a decreased percentage of CD20⁺ cells in one of the three CD20⁺ cases studied. A large Pediatric Oncology Group study of 287 children with ALL demonstrated loss of HLA-Dr expression in 20% and loss of CD10 in 11% (76). Notably, 13 of the 37 pre-B ALL cases studied lost cytoplasmic μ expression, 3 of the 31 T-cell cases converted to a B-lineage phenotype, and 2 of the 144 B-lineage ALL cases converted to a T-lineage phenotype. Finally, van Wering et al. (77) examined 40 cases of childhood ALL at diagnosis and relapse, observing immunophenotypic shifts in approximately two-thirds of the cases. Differences in immunologic marker expression were gen-

erally small. However, TdT expression was lost in 3 of 32 cases of B-lineage ALL, CD10 expression was lost in three, and cytoplasmic μ expression was lost in four. None of the eight T-ALL cases studied lost TdT or CD3 expression, the main markers used to monitor MRD in this disease.

The impact of immunophenotypic changes on MRD monitoring with multiple markers is difficult to predict from the above studies, but it is clearly related to the number of marker combinations that can be applied to each patient. We surveyed, both at diagnosis and at relapse, the immunophenotypic features of children with ALL treated on the three most recent St. Jude protocols, identifying leukemia-associated immunophenotypes at diagnosis in 41 patients whose disease subsequently relapsed (E. Coustan-Smith, F.G. Behm, D. Campana, unpublished data). Because many of these patients' diagnoses had been made several years previously, their immunophenotypic profiles were not as extensive as those currently performed at our institution. Nevertheless, in 33 of the 41 patients (80.5%), at least one leukemia marker combination characterized at diagnosis was retained by the leukemic cells of the recurrent disease. In one of the eight cases that lost its leukemia markers, the cells showed changes in both their immunophenotype and their karyotype at the time of relapse, suggesting the emergence of a new leukemic clone. In all seven other cases, only one leukemia-associated marker combination had been identified at diagnosis, underscoring the importance of using multiple combinations in each case.

Immunophenotypic changes between diagnosis and relapse have also been described in AML. For example, Thomas et al. (78) studied 66 cases of AML at relapse and found an increased expression of CD33 and CD34 and a decreased expression of CD13 and CD15. In the series studied by Macedo et al. (79) 10 of 16 cases showed changes in the expression of at least one marker at the time of relapse, although the authors observed that immunophenotypes useful for MRD monitoring usually remained unaltered.

CLINICAL STUDIES OF MRD BY IMMUNOLOGIC METHODS

Because most T-ALL cases can be studied immunologically, this subtype of leukemia has frequently been included in clinical studies of MRD. Bradstock et al. (46) reported a pioneering investigation in which antisera against TdT and T-cell markers were used with cytocentrifuge preparations to study MRD in patients with T-ALL. Among 18 patients studied, the authors identified six whose remission marrow samples contained 0.5% to 5% nucleated cells with an immunophenotype typical of T-ALL but absent in normal bone marrow cells (T-cell marker in conjunction with nuclear TdT and absence of HLA-Dr). This study provided one of the first indications that leukemic cells could be detected during clinical remission by immunologic methods. The potential usefulness of this approach was demonstrated by Van Dongen et al. (47), who used double-color combinations that included a CD5 and TdT to detect MRD in marrow or peripheral blood samples of 26 T-ALL patients. Their

method predicted 11 of the 12 relapses that occurred in 10 patients 4 to 21 weeks later. By contrast, none of the 16 patients who had no detectable cells positive for CD5 and TdT had relapses during a median follow-up of 43 months. In one of our earlier studies, MRD detection preceded overt relapse by 1 to 6 months in 12 T-ALL patients; of the 13 patients considered to be in clinical and immunologic remission, five subsequently had relapses, one in the CNS (80).

Because of the relative lack of suitable markers, reports of MRD studies in B-lineage ALL have been, until recently, largely anecdotal. For example, Drach et al. (81) monitored MRD in four patients. Of the two patients who had residual cells suggestive of leukemia, one subsequently had a relapse; the other gradually lost the phenotypically abnormal cells and remained in clinical remission until death from infection. The two patients who had negative immunologic MRD studies remained in continuous complete remission with no detectable leukemic cells for 20 and 24 weeks. In our earlier study, clinical relapse occurred in all three MRD-positive B-lineage ALL cases, but in only 2 of the 12 MRD-negative cases, in one of which the relapse was clinically confined to the CNS (80).

The above studies laid the groundwork for prospective investigations of MRD in large series of consistently treated patients. In one recently reported study of this kind, we measured MRD at defined time points during clinical remission in 158 children with ALL treated at our institution (62). We observed residual leukemic cells in 23% of samples studied after the completion of remission induction therapy (6 weeks after diagnosis); the percentage dropped to 17% at week 14, 5% at week 32, 4% at week 56 of continuation therapy, respectively, but in none of the 65 samples studied at the end of therapy (week 120). Detectable residual disease at the end of remission induction therapy was correlated with adverse genetic abnormalities (Philadelphia chromosome and *MLL* gene rearrangements) but not with other presenting features. A positive flow cytometric assay at any point during clinical remission was highly associated with subsequent overt clinical relapse. For example, 3-year cumulative incidence ($\pm$ SE) of relapse was $32.5\% \pm 10.6\%$ in patients with detectable leukemia after remission induction and $7.5\% \pm 4.0\%$ in those who had no detectable leukemia ($P < 0.001$); for tests performed at week 14, these rates were $42.1\% \pm 14.6\%$ and $6.6\% \pm 3.5\%$ ($P < 0.001$). These correlations remained significant after adjusting for adverse prognostic factors. A higher degree of marrow infiltration by leukemic cells ($\geq 0.1\%$) in week 14 samples identified a subset of patients with a dismal outcome. Additional findings corroborating the value of immunologic MRD monitoring were reported by Farahat et al. (82), who used antibodies to TdT, CD10, and CD19 to detect MRD in six of nine patients 5 to 15 weeks before relapse. By contrast, 43 patients who remained in continuous complete remission, with a median follow-up of 23 months, were consistently free of MRD by flow cytometry.

Correlative studies between immunologically detected MRD and treatment outcome have also been extended to

AML. Adriaansen et al. (38) studied 12 patients with TdT⁺ AML during morphologic remission, seven of whom subsequently had one or two relapses (total of 10 relapses). Nine relapses were preceded by a gradual increase in phenotypically abnormal cells. The false-negative results in one case were probably due to a phenotypic shift, since cells were TdT-negative at the time of overt relapse. The remaining five patients stayed in complete remission, with a follow-up of 32 to 46 months. Although small proportions of phenotypically abnormal bone marrow cells ($< 0.1\%$) could be detected in these five patients, the proportions did not increase with serial sampling. Of the five patients with AML in morphologic remission who were studied by Drach et al. (81), three showed persistence or increase of phenotypically abnormal cells, followed by overt relapse. In our earlier study, relapses occurred in all four patients with immunologic evidence of MRD, and in one of three MRD-negative patients (80). In a study by Sievers et al. (83), MRD was detected in 19 of 35 children with AML in first morphologic remission, a finding that correlated with earlier relapse. San Miguel et al. reported the results of sequential studies of MRD in 53 cases of AML (84). The level of MRD at the end of induction and intensification therapy was correlated with subsequent relapse: patients who had more than 5×10^{-3} residual leukemic cells during first remission had a significantly higher rate of relapse than did patients who had fewer than 5×10^{-3} residual leukemic cells (67% versus 20%; $P = 0.002$). At the end of intensification therapy, when a threshold of 2×10^{-3} leukemic cells was used, AML patients composed into two distinct groups with relapse rates of 69% versus 32% ($P = 0.02$).

In general, the above studies indicate that levels of MRD, as detected by immunologic techniques, are associated with risk of relapse. These data strongly suggest that MRD studies should be incorporated into treatment protocols. Single measurements at important time points during therapy (e.g., at the end of remission induction, after consolidation therapy, and at the end of therapy) are informative. A closer monitoring of MRD with repeated sequential sampling during remission (e.g., every three months) may be useful for patients who have had positive MRD findings, but there is no strong evidence that closer monitoring is superior to monitoring at strategic time-points. We are currently assessing whether a more accurate description of early response to treatment, using MRD measurements at earlier time points (e.g., during remission induction therapy) provides useful additional information (Fig. 6).

The use of peripheral blood rather than bone marrow may provide additional opportunities for MRD studies (85). We have begun to explore this possibility by studying paired bone marrow and peripheral blood samples obtained on the date of remission from children with ALL. Our preliminary results indicate that a proportion of cases have detectable leukemic cells in the marrow but not in blood (Fig. 7). Future studies should determine whether

Remission Induction Treatment

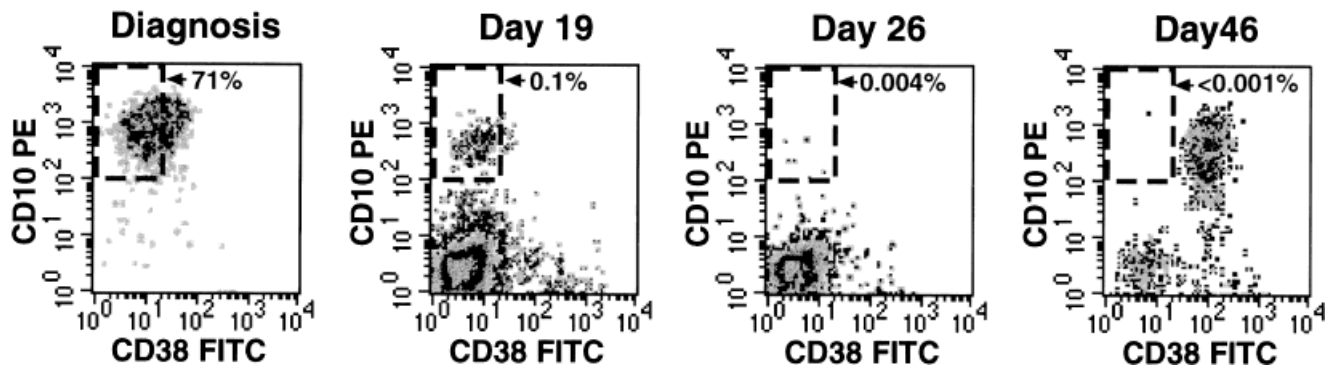


FIG. 6. Monitoring of the kinetics of leukemia disappearance by flow cytometry. Density plots illustrate a serial analysis of MRD in a patient with ALL. At diagnosis, leukemic cells expressed high levels of CD10 and low levels of CD38; 71% of cells fell into an area of the plot which is consistently blank in normal bone marrow (dashed rectangle; far-left panel). Bone marrow samples collected during remission induction therapy did not have leukemic cells detectable morphologically, but still contained immunophenotypically abnormal cells. The percentage of cells with the leukemia-associated immunophenotype within the bone marrow mononucleated cells is indicated.

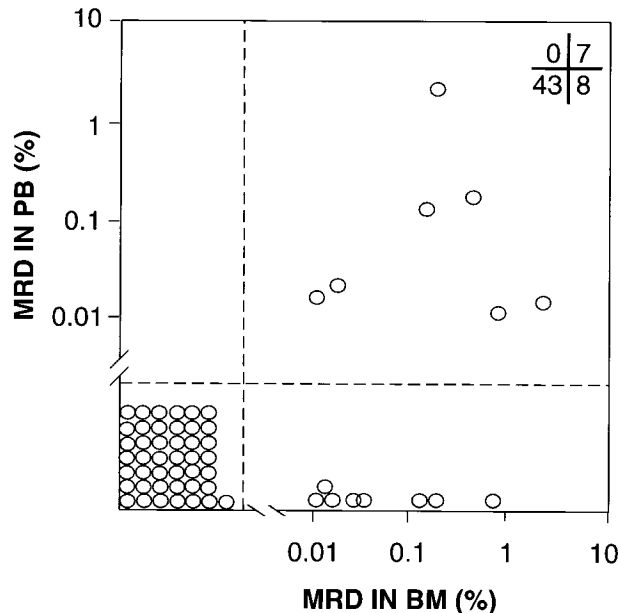


FIG. 7. Immunologic detection of MRD in paired bone marrow and peripheral blood samples from patients with ALL. All samples were in morphologic remission.

these disparities are associated with particular subsets of leukemias and whether tests performed on peripheral blood can be clinically useful.

COMPARISON OF IMMUNOLOGIC METHODS WITH POLYMERASE CHAIN REACTION

Because of its exquisite sensitivity, PCR is a very attractive tool for detecting small numbers of residual leukemic cells (14). In ALL, the primary targets for PCR are clonally rearranged antigen-receptor genes. Reverse-transcriptase (RT)-PCR can also be used to amplify fusion transcripts of chromosomal breakpoints. In AML, only the

latter can be studied because in most cases of AML, antigen-receptor genes retain their germline configuration. The clinical significance of detecting RT-PCR signals in patients who are in clinical remission is still unclear (86–89). By contrast, detection of MRD by PCR amplification of antigen-receptor genes has consistently been correlated with treatment outcome (90–94).

Each method of MRD detection has specific advantages and potential pitfalls (14). For example, immunologic techniques yield a more accurate quantitation of MRD and can discriminate viable from dying cells, whereas PCR may have superior sensitivity. In any case, neither immunologic nor molecular techniques can at present be applied to all patients, which is a prerequisite for the introduction of MRD monitoring in clinical protocols. In ALL, the simultaneous use of immunologic assays and PCR amplification of antigen-receptor genes would allow the study of all patients, but the correlation between measurements by the two techniques is unknown. Immunologic methods are applicable to approximately 80% of cases, whereas PCR is applicable to approximately 85% of cases. We used both immunologic and molecular techniques to study MRD in 46 bone marrow samples collected from children with ALL in clinical remission during therapy (95). Of the 46 samples studied, 8 had more than 1×10^4 leukemic cells by both techniques. Percentages of leukemic cells measured by the two methods correlated well (all paired measurements were within 1 log). Of the remaining 38 samples, 37 had fewer than 1 leukemic cell in 10^4 cells, or undetectable levels of leukemia, whereas one sample had 2.2×10^4 leukemic cells by PCR but no detectable leukemia by flow cytometry. Tests with artificial mixtures of normal and leukemic cells showed that the results of the two methods were highly concordant ($R^2 = 0.966$ by regression analysis). Thus, immunologic and molecular techniques are equally reliable in detecting clinically significant levels of residual leukemia, and can be applied

in tandem for universal monitoring of MRD in childhood ALL.

CONCLUDING REMARKS

According to the recently reported results of large prospective studies (62, 93, 94), measurements of MRD provide a powerful and independent prognostic indicator of treatment outcome in children with ALL, and are likely to have a consequential impact on the clinical management of these patients. The results of MRD studies during the early phases of therapy in this disease are consistent with, and may add to, the predictive value of other measurements of early response to therapy, such as the presence of circulating blast cells at day 7 of therapy (96), the degree of response to prednisone (97), and the morphologic detection of blast cells on days 15 and 21 (98). The results of MRD studies in patients with AML are also strongly suggestive of their clinical value (84). Therefore, it seems reasonable to intensify therapy for those patients who have a slow early response to treatment and have detectable MRD during clinical remission. Conversely, the excellent clinical outcome of MRD-negative cases raises the possibility of using MRD assays to identify candidates for experimenting reductions in treatment intensity.

We still do not know whether early detection of relapse and subsequent changes in therapeutic strategies will improve cure rates, but there is reason to believe that this might be the case. First, it is well established that the tumor burden and the curability of cancer are related. In ALL, for example, a large tumor mass at diagnosis as demonstrated by high leukocyte counts and high serum lactate dehydrogenase activity, is an indicator of poor prognosis (1). Second, the likelihood of the emergence of drug-resistant malignant cells by mutation increases as the number of cell divisions increases, and hence relates to the total tumor burden (99). Thus, timely detection of MRD would identify patients who need more intensive therapy to remain in remission. It may be argued that studies of MRD are unlikely to substantially improve clinical strategies in diseases such as childhood ALL, in which approximately three-fourths of patients can be cured, and for which several risk factors strongly predictive of outcome are already guiding therapy (100). However, known prognostic factors are not 100% predictive, and MRD studies might well complement and enhance their informative value. Therefore, a recently initiated treatment protocol at our institution relies on both presenting features *and* MRD studies (immunologic and molecular) for risk classification of childhood ALL.

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